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Rogers, Douglas G.; Andersen, Arthur A.; and Hunsaker, Breck D., "Lung and nasal lesions caused by a swine Chlamydial isolate in gnotobiotic pigs" (1996). *Papers in Veterinary and Biomedical Science*. 32.
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Lung and nasal lesions caused by a swine Chlamydial isolate in gnotobiotic pigs

Douglas G. Rogers, Arthur A. Andersen, Breck D. Hunsaker

Abstract. The objective of this study was to determine whether a chlamydial isolate recovered from nasal swabs from swine with pneumonia could cause pneumonia and rhinitis in gnotobiotic pigs. The identity of the isolate currently is unknown, but it shares characteristics with *Chlamydia trachomatis*. After propagation in Vero cells and preparation of the inoculum (2.5×10^{10} inclusion-forming units/ml), Chlamydiae were instilled into nostrils (1.0 ml/nostril) and lungs (2.0 ml intralaryngeally) of 15 anesthetized 3-day-old gnotobiotic piglets. Five age-matched gnotobiotic piglets were anesthetized and sham infected with uninfected cell culture lysates. Two treated piglets were moribund and 2 were severely dyspneic prior to necropsy 7 days postinfection (DPI), whereas remaining treated piglets showed mild dyspnea upon exertion throughout the study. All treated piglets developed diarrhea. All treated piglets necropsied 7-21 DPI had extensive consolidation in cranial, middle, and accessory lung lobes; a majority of these piglets also had extensive consolidation in the caudal lobes. Treated piglets necropsied 28 and 35 DPI had a lobular pattern of consolidation in all lung lobes. Histologically, lesions in lungs from treated piglets necropsied 7, 14, and 21 DPI were characterized by bronchointerstitial pneumonia with foci of type II pneumocyte hypertrophy and hyperplasia; pneumocytes and bronchial and bronchiolar epithelial cells were markedly vacuolated. Alveolar macrophages, peribronchitis, peribronchiolitis, and perivascularitis were seen in lungs from treated piglets necropsied 28 and 35 DPI; those necropsied 28 DPI also had foci of lymphohistiocytic and plasmacytic infiltrates. Turbinate lesions in all treated piglets were characterized by mild multifocal lymphoplasmacytic and occasionally neutrophilic rhinitis. Immunohistochemistry detected chlamydial antigen in bronchial and bronchiolar epithelial cells, pneumocytes, and inflammatory cells in treated piglets necropsied 7, 14, and 21 DPI. Positive staining was limited to alveolar macrophages in treated piglets necropsied 28 and 35 DPI. Chlamydial antigen was detected in turbinate epithelial cells at all necropsy intervals. Ultrastructurally, chlamydiae were seen with glycogen particles in vacuoles or free in the cytoplasm of bronchial and bronchiolar epithelial cells and pneumocytes. The results indicated that the chlamydial isolate used in this

Chlamydiae have been associated with pneumonia,¹⁷ enteritis,^{11,13} conjunctivitis,¹⁴ polyarthritis,⁶ pericarditis,¹⁹ perinatal mortality,²² and reproductive disorders^{15,21} in swine; however, the diagnostic and economic significance is unknown. Most of these reports have been based on histopathology, electron microscopy, and in some cases the isolation of chlamydiae. Many of the chlamydial isolates recovered from these disease syndromes are no longer available for study, and only a limited number of these isolates have been characterized.

We recently recovered a chlamydial isolate from nasal swabs from pigs with pneumonia. Chlamydial antigen was detected by immunohistochemistry in lung and turbinate specimens from these pigs; however, known bacterial pathogens also were isolated and were

believed to be the principal causes of the pneumonias. European workers speculate that chlamydiae may cause mild pneumonia in swine and that most naturally occurring chlamydial pneumonias would be complicated by other pathogens. This speculation is based on 1 study in which ovine, bovine, and swine isolates of *Chlamydia psittaci* caused pneumonia in specific-pathogen-free (SPF) pigs.⁵ The biological properties of the swine *C. psittaci* isolates were not described.

The classification of the nasal isolate used in the present study currently is unknown, but it shares characteristics with *C. trachomatis*. Although this isolate is sensitive to sodium sulfadiazine and forms intracytoplasmic inclusions filled with glycogen in cell monolayers, polymerase chain reaction and restriction fragment length polymorphism analyses have not yet confirmed that the isolate is *C. trachomatis*.¹ The objectives of this study were to determine whether this chlamydial isolate could cause pneumonia and rhinitis in gnotobiotic pigs and, if so, to characterize the lesions by immunohistochemistry and light and transmission electron microscopy.

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Received for publication April 18, 1995.

Materials and methods

Isolation of chlamydiae. Vero cells were cultured in Eagle minimum essential medium (EMEM) with Earle balanced salts, 20 mmol of HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 5% fetal bovine serum (FBS)/liter, 5.4 ml glucose/liter, 202 mg glutamine/liter, 2 µg amphotericin B/ml, and 10 µg gentamicin sulfate/ml. The same medium with the addition of 0.5 µg cycloheximide/ml was used as diluent and as maintenance medium following the inoculation of Vero cells with Chlamydiae.

Each cotton-tipped nasal swab specimen collected at necropsy from pigs with pneumonia was immersed in 1.0 ml of sucrose-phosphate-glutamine (SPG) buffer.¹⁶ Swabs were removed from the SPG buffer after a brief period of vigorous agitation, and the buffer specimens were processed for the isolation of Chlamydiae within 24 hr. Buffer specimens were vortexed, and 0.2 ml of each specimen was added to 1.8 ml of the cell culture medium containing cycloheximide. Specimens were again vortexed and then centrifuged under refrigeration at 600 x g for 10 min. The middle 1.0 ml of each specimen was removed and used to inoculate 2 wells (0.1 ml each) on each of 4 96-well dishes containing confluent Vero cell monolayers. The multiwell dishes were centrifuged at 950 x g for 1 hr at 33 C. After centrifugation, 0.1 ml of additional medium was added to each well. The dishes were then incubated for 3-4 days in a humidified 5% CO₂ incubator at 37 C. After incubation, 1 dish was stained by the indirect fluorescent antibody technique using a monoclonal antibody specific for Chlamydial lipopolysaccharide,² and a second dish was stained 5-7 days postinoculation. Wells were read by inverting the plates under an epifluorescence microscope and examining for stained inclusions at 100 x. Negative samples were blind passaged for 1 additional passage. The Chlamydial isolate was designated R33.¹

Preparation of the inoculum. The inoculum was prepared by infecting confluent Vero cell monolayers grown in roller bottles.² Chlamydiae were harvested by disrupting the monolayer with glass beads when 90% cytopathic effect (CPE) was seen. Medium and cells from five 850-cm roller bottles were pooled and then centrifuged at 500 x g for 10 min to remove cellular debris. Chlamydiae were concentrated by centrifugation at 10,000 x g for 1 hr and then suspended in 20 ml of SPG buffer to stabilize.¹⁶ The dosage of inoculum was determined by titration in 24-hr Vero cell monolayers grown in 96-well dishes. After diluting the inoculum 1:4 in sterile phosphate-buffered saline prior to the inoculation of piglets, the final dosage was approximately 2.5 x 10¹⁰ inclusion-forming units/ml. Sham inoculum was prepared in an identical manner from uninfected cell culture lysates.

Gnotobiotic pigs. Gnotobiotic piglets were obtained by closed hysterotomy and were housed in stainless steel isolator units covered by flexible plastic.¹⁰ The isolators were maintained at an ambient temperature of 30 C, and piglets were fed a commercial milk substitute^a 3 times daily. Fecal swab specimens were collected from each piglet prior to experimental inoculation and inoculated onto sheep blood agar for aerobic and anaerobic^b bacteriologic culture and onto tergitol-7 agar for aerobic culture.

Inoculation. Twenty 3-day-old gnotobiotic piglets were

randomly allotted into isolators. Piglets were anesthetized with tiletamine HCl and zolazepam HCl^c by intramuscular injection (0.088 ml/kg)¹⁸ prior to experimental inoculation. Fifteen piglets were inoculated with chlamydiae, and 5 piglets served as sham-infected controls. A sterile 20-gauge 2.54 cm needle with an attached sterile syringe was used to slowly instill 2 ml of inoculum or sham inoculum into the lungs of each piglet by the intralaryngeal route. A sterile, 22-gauge ball-tip rodent gavage needle with an attached sterile syringe, inserted past the medial alae, was used to slowly instill 1 ml of inoculum or sham inoculum into each nostril of each piglet.

Necropsy, histopathology, and electron microscopy. Piglets were monitored for anorexia, dyspnea, hyperpnea, coughing, sneezing, and nasal discharge twice daily. Necropsies were done when treated piglets displayed prominent clinical symptoms or at predetermined intervals postinfection. Piglets were anesthetized prior to euthanasia by electrocution.

Lung, liver, duodenum, jejunum, ileum, colon, and nasal turbinates from all piglets were fixed in neutral-buffered 10% formalin, routinely processed, embedded in paraffin, sectioned at 4 µm, stained with hematoxylin and eosin (HE), and examined by light microscopy. Nasal turbinate specimens were decalcified⁸ prior to processing for light microscopy. Specimens of lung and nasal mucosa from piglets necropsied 7 days postinfection (DPI) were fixed in neutral phosphate-buffered 3% glutaraldehyde, postfixed in osmium tetroxide, dehydrated in graded ethanols, infiltrated with propylene oxide, and embedded in epoxy resin. Semithin (1 µm) sections were cut and stained with toluidine blue. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope.^d

Reisolation of chlamydiae. Lung specimens from all piglets were ground as a 1:10 suspension in the tissue culture medium with antibiotics. After centrifugation at 500 x g for 10 min, the supernatant was diluted 1:5 in the tissue culture medium with antibiotics and used to inoculate Vero cell monolayers as previously described for the processing of swab specimens. Nasal swab specimens from all piglets and fecal swab specimens from treated piglets were processed as previously described.

Bacteriology and mycoplasmaology. Lung tissue from each piglet was cultured aerobically on Columbia colistin-nalidixic acid (CNA) and MacConkey (MAC) agars and on sheep blood agar (SBA) with a nurse streak of *Staphylococcus aureus*. Additional specimens from each piglet that were cultured aerobically included nasal swab (CNA, MAC, SBA), liver (MAC, SBA, tetrathionate broth), jejunum, ileum, and colon (SBA, tergitol-7 agar, tetrathionate broth). Intestinal specimens also were cultured anaerobically^b on SBA.

Nasal swab^c and lung specimens from each piglet were processed to inoculate liquid and solid Friis, M96, and Special M96 media for the isolation of mycoplasmas.^f

Virology. Lung, tonsil, and spleen were collected from each piglet for the isolation of pseudorabies virus (PRV), and lung and spleen were collected for the isolation of porcine parvovirus (PPV). Tissue specimens were homogenized in Dulbecco's minimal essential medium with gentamicin and centrifuged at 1,200 r-pm (400 x g) for 20 min. Supernatants

were inoculated onto 24-hr cultures of porcine fallopian tube cells and primary porcine lung cells in Bell tubes. Inoculated cell cultures were incubated at 37 C and examined daily for 7 days for evidence of CPE. If CPE was not seen after 7 days, inoculated cell cultures were frozen and then thawed, and supernatants were transferred to fresh 24-hr cell cultures in Leighton tubes. Leighton tubes were incubated at 37 C, and cultures were examined daily for 7 days for CPE. Cultures were considered negative if CPE was not seen.

Lung, tonsil, spleen, thymus, and serum were collected from each piglet for the isolation of porcine reproductive and respiratory syndrome virus (PRRSV). Tissue specimens were homogenized in EMEM containing 3 µg/ml amphotericin B, 75 µg/ml gentamicin, 250 units/ml penicillin G, and 200 µg/ml streptomycin. Tissue homogenates were centrifuged under refrigeration for 20 min at 1,500 x g, and 1-2-ml aliquots of each supernatant were passed through a disposable 0.45 µm filter. Tissue supernatants and 0.5-ml aliquots of each serum sample were inoculated into Leighton tubes containing confluent MARC-145 cells.⁸ Inoculated cultures were incubated for 18-24 hr at 37 C. After incubation, the growth medium containing the inoculum from each tube was discarded, and 9-10 ml of fresh EMEM containing gentamicin and 4% FBS was added to each tube. Inoculated cultures were incubated for 9 days at 37 C and examined every other day for CPE. After incubation, inoculated cell cultures were frozen and then thawed. Growth medium with cells (2 ml) was then transferred to fresh confluent MARC-145 cell cultures from which the growth medium had been discarded and to which fresh medium containing 4% FBS had been added. The second passage cultures were then incubated 7-9 days at 37 C and examined for CPE. Cultures were considered negative if CPE was not seen.

Jejunum and ileum from treated piglets were examined for rotavirus (RV) and transmissible gastroenteritis virus (TGEV) by fluorescent antibody techniques. Feces from treated piglets were examined for viruses by negative contrast electron microscopy.

Immunohistochemical procedure. Primary polyclonal antiserum was obtained from rabbits that had been hyperimmunized with ovine abortion strains B577 and OSP of *C. psittaci*. The antiserum was purified by column chromatography^h and used as primary antibody at a dilution of 1:750 in Tris buffer containing 2.0% normal swine serum. A dilution of 1:750 was used because of reduced nonspecific binding to tissue sections.

Tissue specimens were fixed, processed, and embedded in paraffin as previously described. Sections of lung and nasal turbinate from all piglets and sections of jejunum and ileum from selected treated piglets were cut at 4 µm and attached to glass microslides that had been precoated with Vectabond adhesive.ⁱ Sections were then deparaffinized for 15 min in xylene and subsequently rehydrated for 20 min in graded ethanols containing 0.1% concentrated HCl. Sections were then washed twice in 0.01 M Tris buffer (pH 7.6) containing 0.001% Tween 20 (Tris-Tween). After 1 wash in Tris-Tween buffer at 37 C, sections were placed in Tris buffer containing 0.05% protease XIV for 15 min at 37 C. Sections were then washed twice in Tris-Tween buffer at room temperature.

All steps in the staining protocol were done at room tem-

perature. Nonspecific adherence of immunoglobulin was minimized by covering the sections with 20% normal goat serum in Tris buffer for 30 min in a humidified chamber. Excess normal goat serum was removed by inverting the slide and blotting. Primary antibody was applied to sections for 90 min in a humidified chamber. After 2 washes in Tris-Tween buffer, sections were covered with biotinylated goat anti-rabbit IgG (heavy and light chain-specific)^j diluted 1:200 in Tris buffer for 60 min in a humidified chamber, followed by 2 washes in Tris-Tween buffer. Sections were then covered with a freshly prepared avidin-biotin-alkaline phosphatase solutionⁱ for 30 min in a humidified chamber. After 2 washes in Tris-Tween buffer, sections were covered for 30 min with a freshly prepared substrate solution (Vector Red)ⁱ containing levamisoleⁱ in a humidified chamber. Sections were then washed in ultrapure water for 10 min.

Sections were stained for 5 min with Gill's hematoxylin, washed twice in Tris-Tween buffer, dehydrated in graded ethanols, and cleared in xylene. Coverslips were mounted with water-insoluble medium, and sections were examined by light microscopy.

Sections of pig small intestine with villi heavily colonized by enterotoxigenic *Escherichia coli* and sections of canine lung from which a pure culture of *Bordetella bronchiseptica* had been isolated in large numbers were stained to determine the potential for cross-reactivity with other gram-negative bacteria. Performance controls incorporated into each staining procedure included sections of ovine placenta with large numbers of *C. psittaci* (positive tissue control), lung and nasal turbinate from the age-matched sham-infected piglets (negative tissue control), and the substitution of normal rabbit serum diluted 1:750 in Tris buffer with 2.0% normal swine serum for anti-*Chlamydia* antibody in the staining procedure (negative serum control).

Results

Clinical symptoms and gross lesions. The necropsy schedule is shown in Table 1. Rectal temperatures of sham-infected piglets were 38.6-39.0 C throughout the study, and these piglets did not exhibit any clinical symptoms. Two treated piglets became moribund, and 2 became severely dyspneic prior to necropsy at 7 DPI. These 4 piglets had elevated rectal temperatures (40.0-40.4 C) 4-5 DPI, and temperatures remained elevated in the 2 dyspneic piglets just prior to necropsy. Rectal temperatures in the 2 moribund piglets were subnor-

Table 1. Necropsy schedule for piglets infected with chlamydia and piglets sham infected.

Days postinfection	No. piglets necropsied	
	Infected	Sham infected
7	4	1
14	4	1
21	4	1
28	2	1
35	1	1

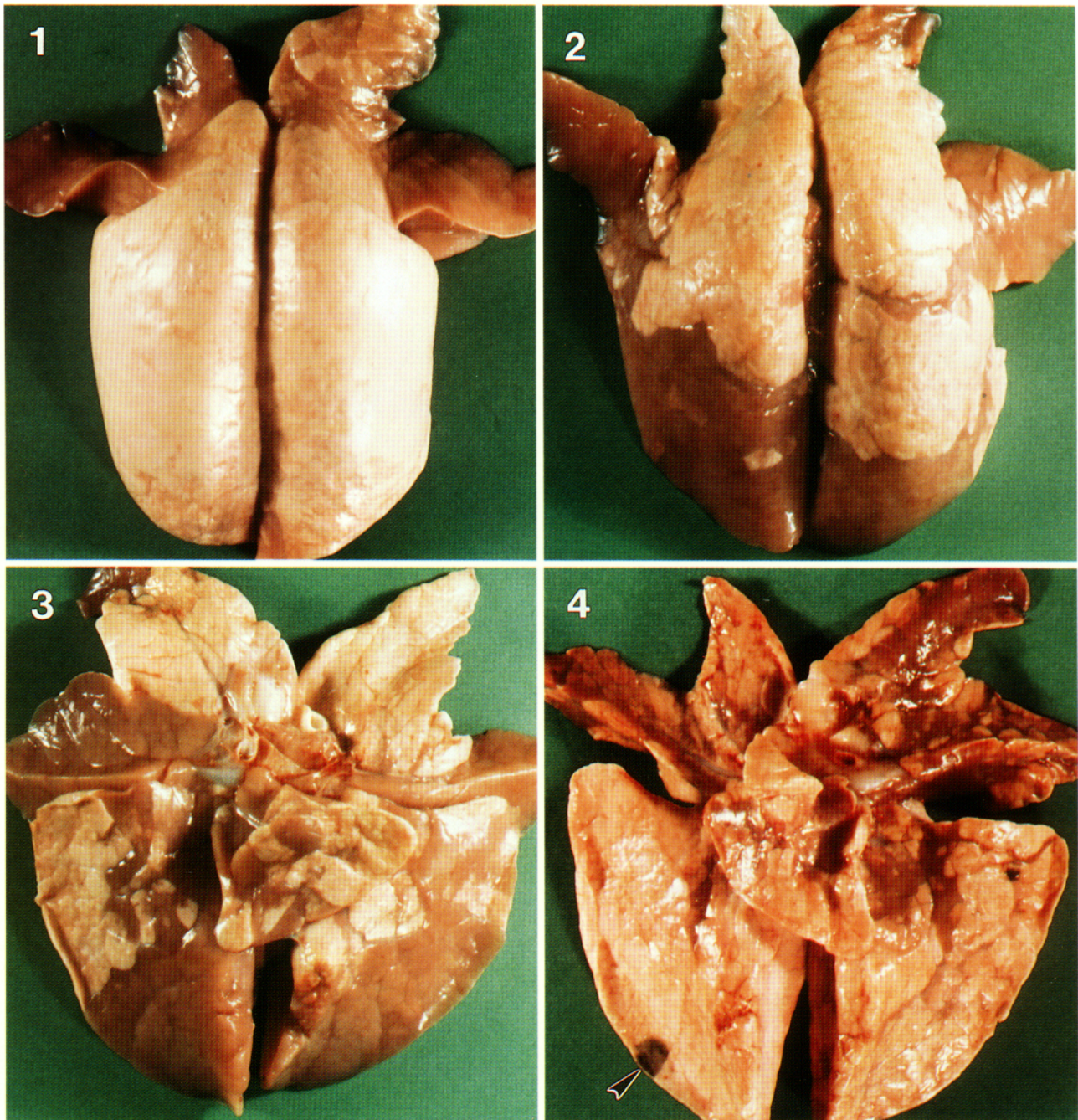


Figure 1. Gross lung lesions typical of those seen in 5 piglets necropsied 7–21 days postinfection. There is consolidation of cranial and middle lung lobes. Right cranial lobe has a lobular pattern of consolidation.

Figure 2. Gross lung lesions typical of those seen in 7 piglets necropsied 7–21 days postinfection. There is consolidation of middle lung lobes and extensive foci of consolidation in caudal lobes. Right cranial lobe has small focus of consolidation.

Figure 3. Gross lung lesions typical of those seen in 7 piglets necropsied 7–21 days postinfection. Ventral aspect of lungs shown in Fig. 2.

Figure 4. Ventral aspect of gross lung lesions typical of those seen in piglets necropsied 28 and 35 days postinfection. There is patchy, lobular consolidation in all lung lobes and a focus of melanosis in right caudal lobe (arrowhead).

mal (36.2–36.6 °C) just prior to necropsy. Remaining treated piglets exhibited mild dyspnea upon exertion throughout the study, and they did not become inappetent. Compared with sham-infected piglets, these

treated piglets had slightly elevated rectal temperatures (39.4–39.7 °C) 4–5 DPI through 21 DPI.

At necropsy, all treated piglets were thin and had roughened hair coats. Those necropsied 7, 14, and 21

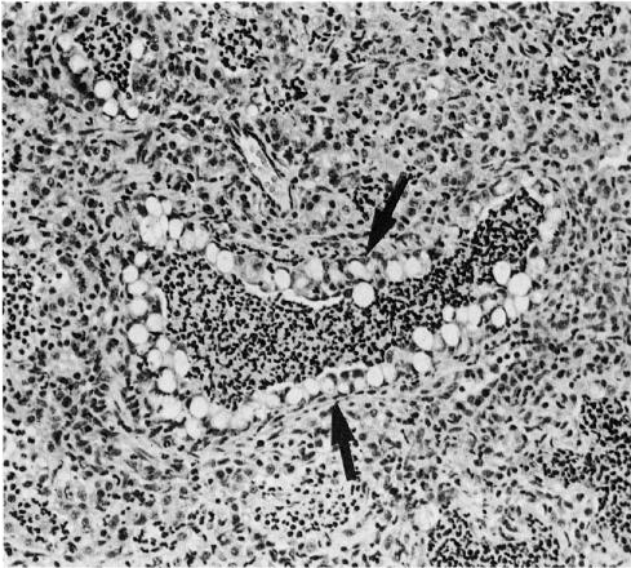


Figure 5. Lung; piglet necropsied 7 days postinfection. There is extensive vacuolization of bronchiolar epithelial cells (arrows). The bronchiolar lumen is filled with neutrophils. HE.

DPI had hydropericardium and watery colonic contents. All treated piglets had gross lung lesions that were dark tan to plum colored and firm. Five of the 12 treated piglets necropsied 7-21 DPI had extensive bilateral consolidation of the cranial and middle lung lobes (Fig. 1) and patchy lobular consolidation of the accessory lobe. The remaining 7 treated piglets necropsied 7-21 DPI had extensive bilateral foci of consolidation in the caudal lobes, and foci of consolidation of various sizes in the other lobes (Figs. 2, 3). Treated

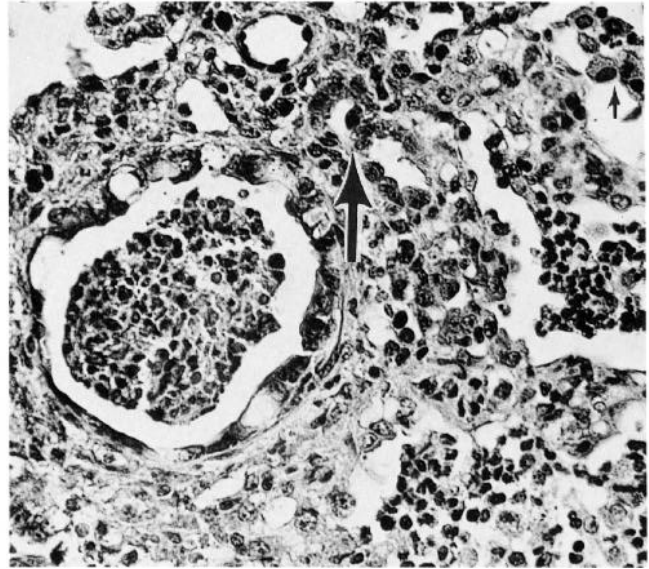


Figure 7. Lung; piglet necropsied 7 days postinfection. Small focus of hypertrophic and hyperplastic type II pneumocytes (large arrow). The bronchiole is partially lined by flattened epithelium, occasional bronchiolar epithelial cells have vacuoles, and the lumen is filled with neutrophilic debris. Neutrophils and occasional foamy macrophages (small arrow; upper right) are in alveolar spaces. HE.

piglets necropsied 28 and 35 DPI had patchy foci of lobular consolidation in all lobes (Fig. 4). There were no gross lesions in turbinates of treated piglets, and there were no gross lesions of any kind in the sham-infected piglets.

Histopathology. Lung from treated piglets necropsied 7 DPI was characterized by focally extensive bron-

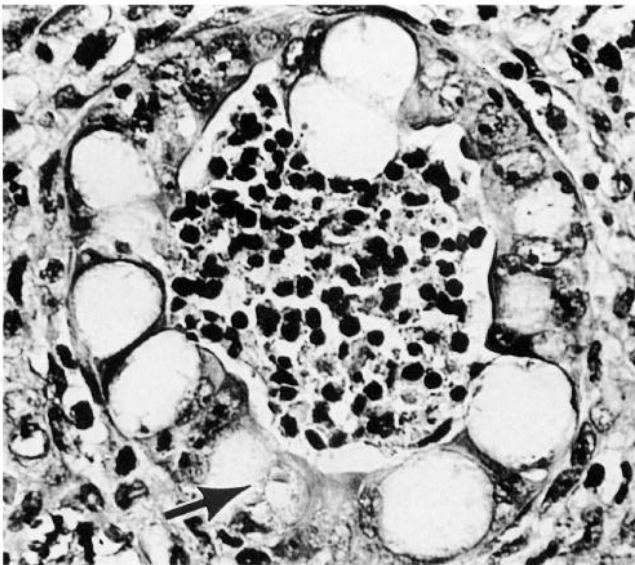


Figure 6. Lung; piglet necropsied 7 days postinfection. Bronchiolar epithelial cells have large vacuoles. Coccoid organisms are barely discernible in vacuoles (arrow). The bronchiolar lumen is filled with neutrophilic debris. HE.

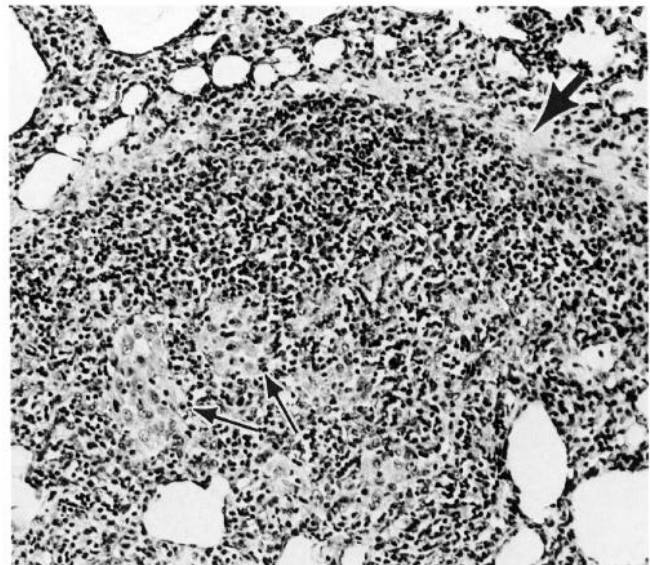


Figure 8. Lung; piglet necropsied 28 days postinfection. Lung subjacent to interlobular septum (large arrow) has been infiltrated by large macrophages (small arrows) and other mononuclear inflammatory cells. HE.

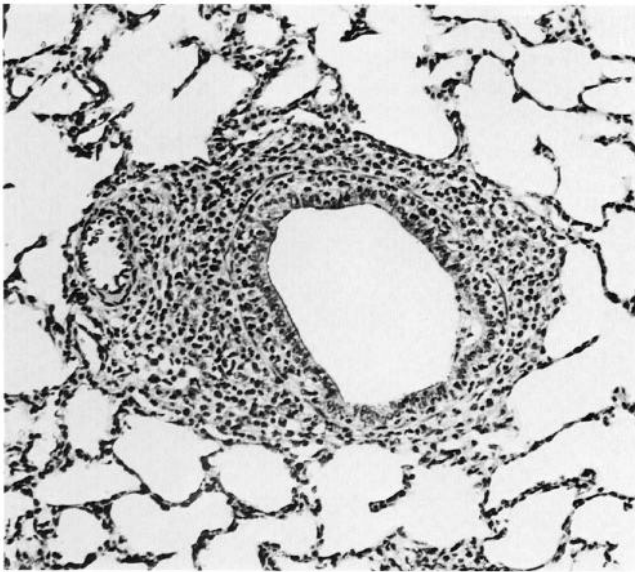


Figure 9. Lung; piglet necropsied 35 days postinfection. Note peribronchiolar and perivascular cuffs of mononuclear inflammatory cells. HE.

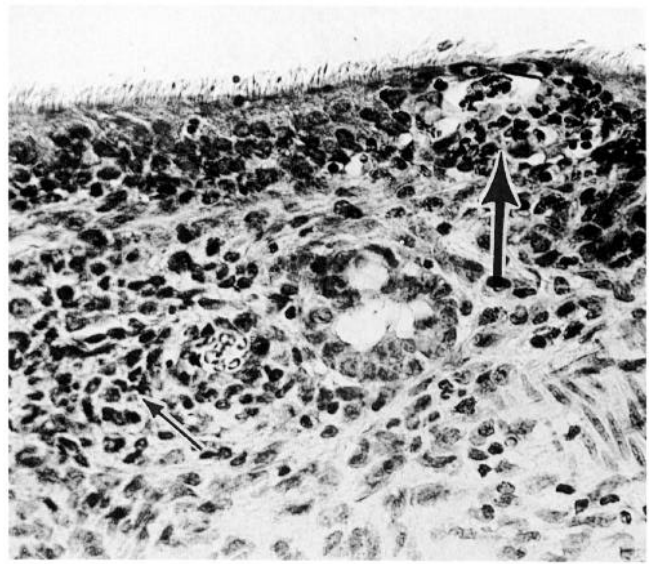


Figure 10. Nasal turbinate mucosa; infected piglet. Intra-epithelial cleft is filled with neutrophils (large arrow), and there are small numbers of inflammatory cells in the propria-submucosa (small arrow).

chointerstitial pneumonia. Scattered bronchi and bronchioles were filled with neutrophils and occasional macrophages. Bronchi had intraepithelial clefts that were filled with neutrophils, and occasional epithelial cells were markedly vacuolated. There was extensive vacuolization of bronchiolar epithelial cells (Fig. 5), and lightly basophilic coccoid organisms were occasionally seen in the vacuoles (Fig. 6). Bronchioles filled with inflammatory cells were occasionally lined by flattened epithelium or were devoid of lining epithelium. Alveolar septa were thickened by macrophages, lymphocytes, occasional neutrophils, and rare eosinophils, and there were extensive foci of type II pneumocyte hypertrophy and hyperplasia (Fig. 7); occasional hypertrophic pneumocytes were markedly vacuolated. Alveolar spaces were filled with neutrophils, foamy macrophages (Fig. 7), rare eosinophils, inflammatory

cell debris, and proteinaceous material (edema). Scattered bronchi, bronchioles, and blood vessels in some sections were surrounded by thin cuffs of lymphocytes, plasma cells, and macrophages.

Bronchointerstitial pneumonia was seen in lungs from treated piglets necropsied 14 and 21 DPI. However, there were less extensive foci of type II pneumocyte hypertrophy and hyperplasia and considerably fewer vacuolated bronchial and bronchiolar epithelial cells than in treated piglets necropsied 7 DPI. Alveolar exudate in sections from the 14- and 21-DPI piglets was predominantly neutrophilic and histiocytic; however, in scattered foci it was lymphohistiocytic.

Foci of various sizes of large foamy macrophages, lymphocytes, and plasma cells were seen in lung from treated pigs necropsied 28 DPI. Although random in distribution, these foci were often subjacent to pleura

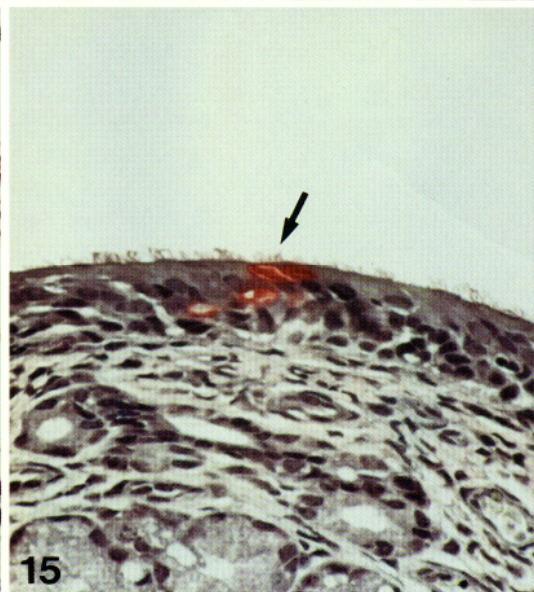
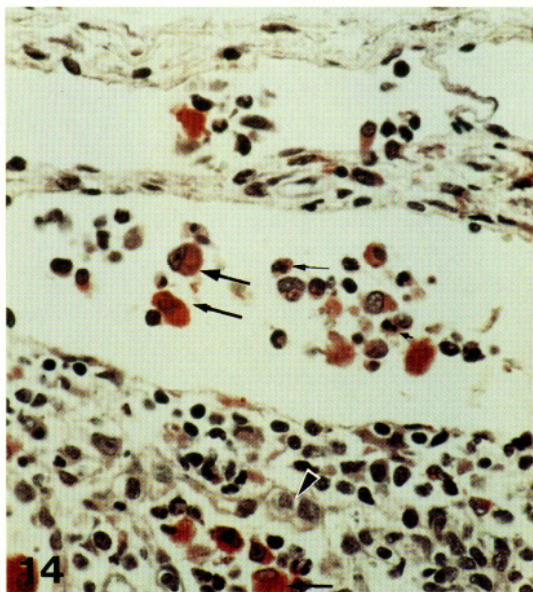
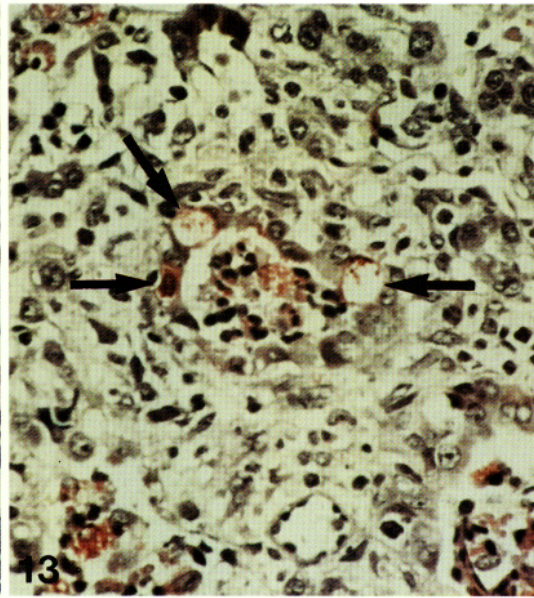
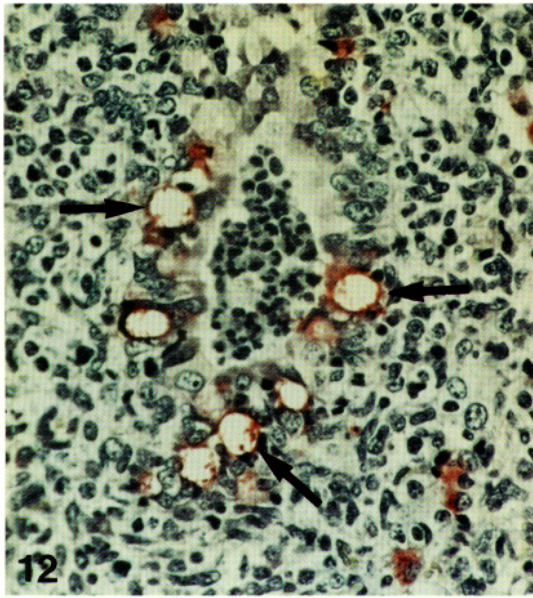
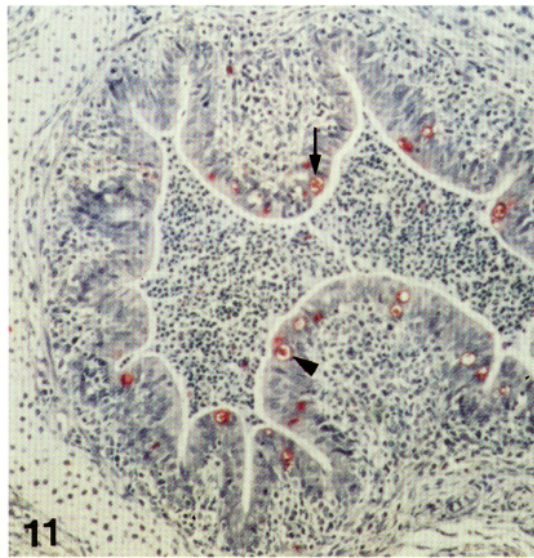
Figure 11. Lung; piglet necropsied 7 days postinfection. Chlamydial antigen (red reaction product) appears in scattered bronchial epithelial cells. Note Chlamydial antigen in vacuoles (large arrows). Avidin-biotin-alkaline phosphatase stain, hematoxylin counterstain.

Figure 12. Lung; piglet necropsied 7 days postinfection. Chlamydial antigen appears in large vacuoles in bronchiolar epithelial cells (arrows). Avidin-biotin-alkaline phosphatase stain, hematoxylin counterstain.

Figure 13. Lung; piglet necropsied 7 days postinfection. Chlamydial antigen appears in pneumocytes (arrows) in focus of type II pneumocyte hypertrophy and hyperplasia. Note large vacuoles in type II pneumocytes. There is faint staining in neutrophils surrounded by the type II pneumocytes. Avidin-biotin-alkaline phosphatase stain, hematoxylin counterstain.

Figure 14. Lung; piglet necropsied 7 days postinfection. Note intense positive staining of macrophages (large arrows) in alveolar spaces and in interlobular lymphatics and faint staining of neutrophils (small arrows). Hypertrophic and hyperplastic type II pneumocytes partially line an alveolar space filled with macrophages and neutrophils (arrowhead). Avidin-biotin-alkaline phosphatase stain, hematoxylin counterstain.

Figure 15. Nasal turbinate mucosa; infected piglet. Chlamydial antigen appears in epithelial cells (arrow).



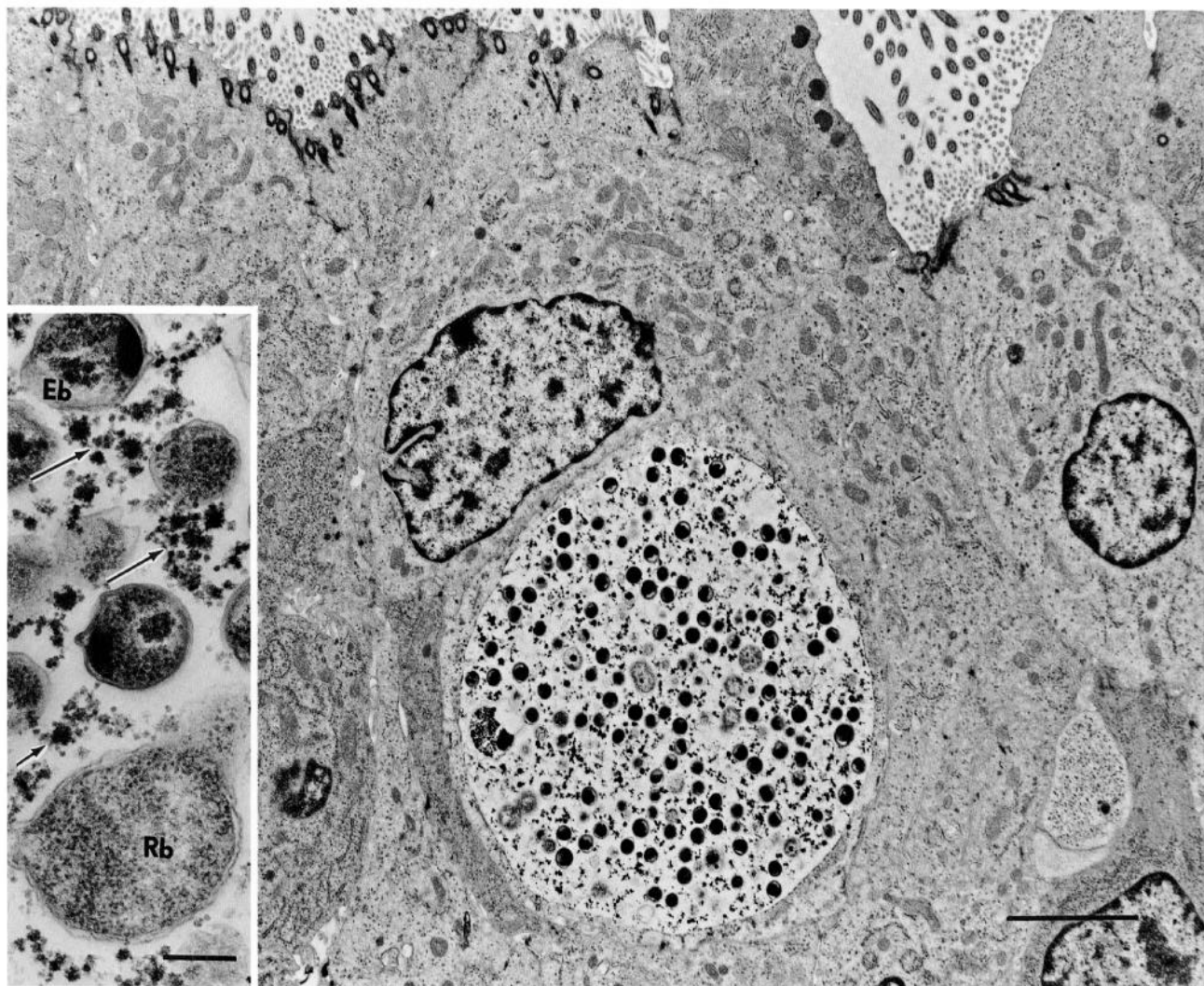


Figure 16. Lung; piglet necropsied 7 days postinfection. Intracytoplasmic vacuole is filled with Chlamydiae and flocculent electron-dense material in bronchial epithelium. Bar = 3.0 μ m. *Inset:* Flocculent electron-dense material in vacuole has ultrastructural characteristics of glycogen (arrows). Rb = chlamydial reticulate body; Eb = elementary body. Bar = 0.25 μ m.

and interlobular septa (Fig. 8). In lung from the 28- and 35-DPI piglets, scattered bronchi, bronchioles, and blood vessels were surrounded by thin cuffs of lymphocytes, plasma cells, and macrophages (Fig. 9). There also were multiple foci of alveolar septa thickened by macrophages and lymphocytes, and large foamy macrophages were scattered within alveolar spaces.

Nasal turbinate mucosa from all treated piglets had scattered intraepithelial clefts that were filled with neutrophils (Fig. 10), and occasional epithelial cells were markedly vacuolated. Small focal infiltrates of lymphocytes, plasma cells, and occasional eosinophils were widely scattered within the propria-submucosa (Fig. 10).

Ileum from 3 treated piglets necropsied 7 DPI and from 3 necropsied 14 DPI was characterized by mild multifocal villus atrophy. There were no lesions in liver

from treated piglets, and there were no lesions in any of the tissues from the sham-infected piglets.

Immunohistochemistry. Evaluation of performance controls indicated that the immunohistochemical procedure was valid. Chlamydial antigen was abundant in lung from the treated piglets necropsied 7 DPI and was seen in bronchial epithelial cells (Fig. 11), bronchiolar epithelial cells (Fig. 12), pneumocytes (Fig. 13), and inflammatory cells. Positive staining was especially intense in macrophages in alveolar spaces and in interlobular lymphatics (Fig. 14), whereas staining of macrophages in alveolar septa was sparse. Considerably fewer bronchial epithelial cells, bronchiolar epithelial cells, and pneumocytes had antigen in sections from the 14- and 21-DPI piglets. Positive staining of macrophages and occasional neutrophils in alveolar spaces and in interlobular lymphatics was consistently

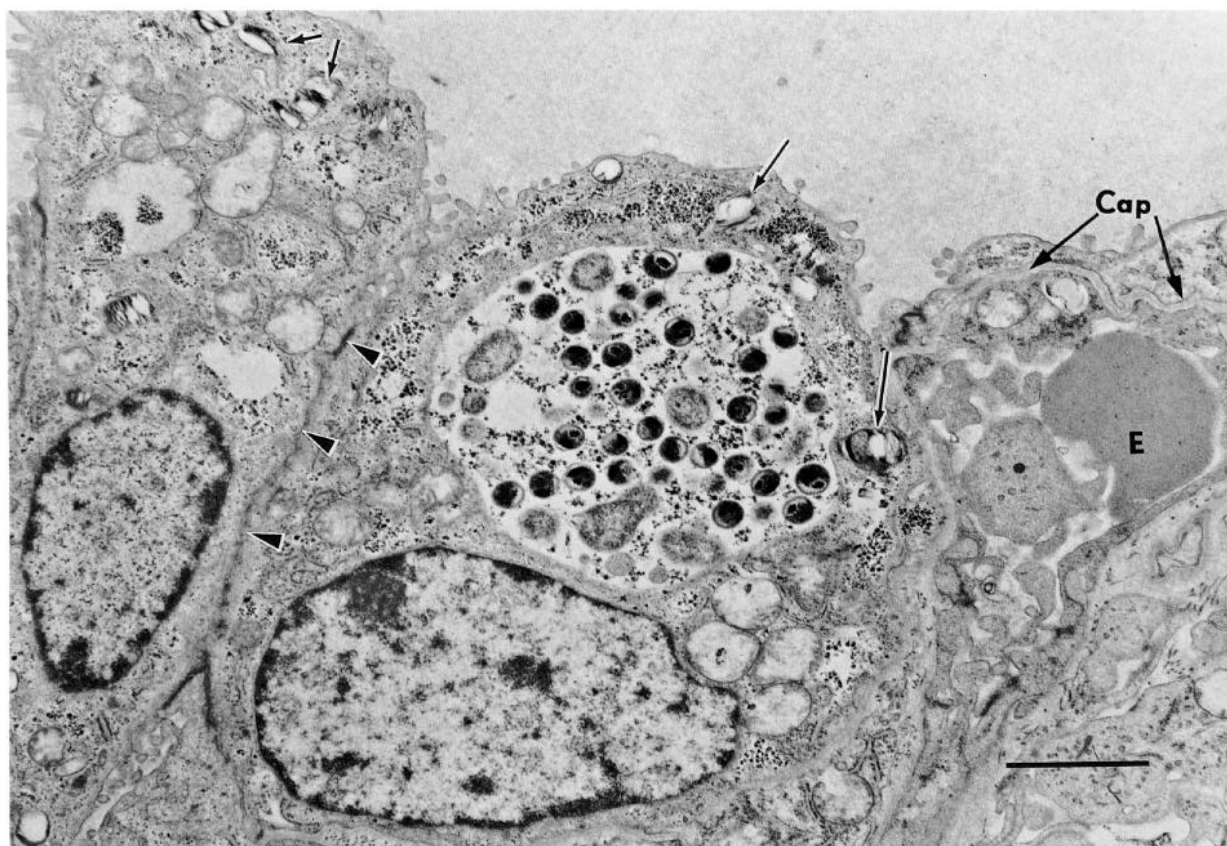


Figure 17. Lung; piglet necropsied 7 days postinfection. Type II pneumocyte has intracytoplasmic vacuole filled with chlamydiae and glycogen. Cell borders adjacent type II pneumocyte (arrowheads). There are characteristic lamellar inclusions in cytoplasm of type II pneumocytes (arrows). Note alveolar septal capillary (Cap) with intraluminal erythrocyte (E). Bar = 2.0 μ m.

seen in sections from the 14- and 21-DPI piglets, whereas sparse staining of alveolar macrophages only was seen in sections from the 28- and 35-DPI piglets. Sparse positive staining of nasal epithelial cells was seen in turbinate from all treated piglets (Fig. 15). Examination of jejunum and ileum (with villus atrophy) from 2 selected treated piglets, both necropsied 7 DPI, revealed chlamydial antigen in villus enterocytes.

Transmission electron microscopy. Ultrastructural examination of lung specimens from the treated piglets necropsied 7 DPI revealed chlamydiae, together with glycogen particles, in the cytoplasm of bronchial epithelial cells (Fig. 16), bronchiolar epithelial cells, and pneumocytes (Fig. 17). Intracytoplasmic chlamydiae were most often in membrane-bound vacuoles, but occasionally they were free in the cytoplasm. All morphologic forms of the chlamydial developmental cycle were seen. Dead and degenerate chlamydiae occasionally were seen in the cytoplasm of macrophages and neutrophils in airway lumina and alveolar spaces. Chlamydiae were not found ultrastructurally in turbinate specimens.

Microbiology. Chlamydiae were reisolated from lung and nasal swab specimens from treated piglets nec-

ropsied 7-21 DPI and from 1 of the 2 treated piglets necropsied 28 DPI. Chlamydiae were not isolated from sham-infected piglets. Bacteria were not isolated from nasal swab or tissue specimens from treated or sham-infected piglets necropsied 7-21 DPI. However, *Bacillus* sp. was isolated from intestinal specimens from treated and sham-infected piglets necropsied 28 and 35 DPI. Mycoplasmas, PRV, PPV, or PRRSV were not isolated from specimens from treated or sham-infected piglets. Fluorescent antibody tests for RV and TGEV done on jejunum and ileum from treated piglets were negative, and viruses were not seen in feces from the these piglets.

Discussion

The results indicated that the swine chlamydial isolate (R33) used in this study is a pathogen in gnotobiotic pigs. The classification of this isolate currently is unknown. The isolate is sensitive to sodium sulfadiazine and forms inclusions filled with glycogen in cell monolayers, which would suggest that it is *C. trachomatis*.¹² The fact that intracytoplasmic vacuoles filled with glycogen particles and chlamydiae were seen ultrastructurally in lung from pigs in this study might

further support this hypothesis. Although the *C. trachomatis* classification traditionally has included human chlamydial isolates and 1 mouse isolate,¹² a *C. trachomatis* isolate was recently recovered from an inapparent intestinal infection in swine.⁴ Work is underway to further characterize and identify the isolate used in the present study.

Gross lung lesions were nonspecific and could mimic other bacterial or mixed infections. Lesions involving only cranial, middle, and accessory lobes as seen in several principals resembled those described for mycoplasmosis.⁷ With the exception of large vacuoles in pneumocytes and in epithelial cells lining bronchi, bronchioles, and turbinates, histologic lesions in lung and nasal turbinate also were nonspecific. The ability to cause large vacuoles in host cells may be a characteristic of the isolate used in the present study and may or may not be a characteristic of other isolates. Vacuolization of host cells was not described in an earlier European study in which SPF pigs were infected with ovine, bovine, and swine *C. psittaci* isolates.⁹

With the exception of 2 moribund and 2 severely dyspneic treated piglets necropsied 7 DPI, remaining treated piglets had few respiratory symptoms other than mild dyspnea upon exertion. The relative lack of respiratory symptoms in these remaining piglets was surprising because lung lesions were often extensive at necropsy, especially in the piglets necropsied 14 and 21 DPI. Chlamydiae probably caused the diarrhea seen in the treated piglets; no other known infectious agents were identified. In addition, chlamydiae were consistently isolated from feces of diarrheic treated piglets and immunohistochemical staining, although done on a limited basis, detected chlamydial antigen in enterocytes lining atrophic ileal villi. Chlamydiae have been associated with enteritis in swine,^{11,13} but their role as true pathogens has never been established.

The diagnosis of swine chlamydial infections is relatively rare, especially in the United States, possibly because of uncertainty regarding the role of chlamydiae as pathogens and because most diagnostic laboratories do not routinely examine tissue specimens from swine for the presence of chlamydiae. There also may be a tendency to incriminate known pathogens in certain diseases of swine, thus ignoring a possible synergistic role for chlamydiae. Serologic surveys done in Europe suggested that exposure to chlamydiae is widespread among swine.^{3,20} In limited serologic testing using a microimmunofluorescence test, > 90% of pigs tested had antibodies to chlamydiae (Anderson et al., unpublished data). Additional studies are necessary to clarify the role of chlamydiae in the pathogenesis of swine diseases. Progress will depend on the isolation of chlamydiae from various disease syndromes, characteriza-

tion of these isolates, and the subsequent reinoculation of pigs.

Acknowledgements

We thank Linda Homung, Wendy Hambly, Everine Breden, and Tom Bargar for technical assistance. This work was supported by funds from the Department of Veterinary and Biomedical Sciences, University of Nebraska. This article is published as paper no. 11098, Agricultural Research Division, Institute of Agriculture and Natural Resources, University of Nebraska-Lincoln.

Sources and manufacturers

- a. SPF-Lac, Borden, Norfolk, VA.
- b. BBL GasPak, Becton Dickinson, Cockeysville, MD.
- c. Telazol, Ft. Dodge Laboratories, Ft. Dodge, IA.
- d. Philips 201, Eindhoven, The Netherlands.
- e. Cultureswab Transport System, Difco, Surrey, UK.
- f. National Veterinary Services Laboratories, Ames, IA.
- g. Dr. Merwin Frey, National Veterinary Services Laboratories, Ames, IA.
- h. CM Affi-Gel Blue, Bio-Rad Laboratories, Richmond, CA.
- i. Vector Laboratories, Burlingame, CA.
- j. Sigma Chemical Co., St. Louis, MO.

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